Peroxisomal ascorbate peroxidase gene induced by high temperature stress and a transgeneic plant exhibiting thermotolerance

BACKGROUND OF THE INVENTION

1. Field of the invention

[0001] This invention relates to peroxisomal ascorbate peroxidase gene from barley, which is a novel gene induced by high temperature stress and a transgenic plant exhibiting improved thermotolerance produced by incorporating said gene.

2. Description of Related Art

[0002] Food crisis is expected to occur in the near future, caused by increase of population and alteration of environment. Therefore, in order to cope with the problem, there is a great demand on development of a agricultural technique that enables efficient production of crops. The amount of agricultural production tends to be effected by various environmental stresses. It reserves to be a serious problem in the field of agriculture. Therefore production of a plant, exhibiting resistance to various environmental stress, is needed. Especially, anathermal of the earth is a serious problem. Therefore, production of a plant having improved thermotolerance reserves to be great value.

[0003] Until now, for the production of a transgenic plant having improved thermotolerance by a genetic engineering technique, a gene encoding heat shock protein, induced by high-temperature stress, have been utilized for such purpose. Furthermore in order to attain above purpose, small amount of glycine betaine, which is a suitable solute to prevent enzyme denaturation under heat stress, was also bio-synthesized in a plant body. Moreover, it has been recognized that genes, involved in decomposing of active oxygen molecules, are important for resistance to environmental stress. Using such

genes, resistance to photo-oxidation injury, resistance to salt stress or resistance to aridity stress was rendered to a plant by incorporating said gene involved in resistance to environmental stress. Despite of it, there have been no report describing on imparting of thermotolerance, achieved by incorporating genes involved in decomposition of active oxygen molecules described above.

SUMMARY OF THE INVENTION

[0004] The inventor has remarked importance of genes involved in decomposition of active oxygen molecules and challenged on imparting thermotolerance to a plant by incorporating a gene involved in decomposition of active oxygen molecules, not by conventional techniques of incorporating a gene encoding heat shock protein. That is, it is an object of this invention to develop a novel technique which can impart thermotolerance to a plant by incorporating a gene involved in defense to oxidative stress. In the concrete, it is an object of this invention to obtain a novel gene useful for such purpose and determine base sequence of the gene.

[0005] High temperature stress is one of the main factors constraining the growth and productivity of plants. Living organisms respond rapidly to high temperatures by metabolic changes that involve complex reprogramming of cellular activities. These changes must help to protect the essential structures and functions of cells against damage caused by the stress. It is known that heat shock can result in an oxidative stress, which induces genes involved in the oxidative stress defense. Under heat stress, excessive active oxygen species (AOS) such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals are formed which cause oxidative damage of cell constituents.

[0006] A major hydrogen peroxide detoxifying system in plant chloroplast and cytosol is the so-called ascorbate glutathione cycle, in

which APXs (ascorbate peroxidases) are the key enzymes. APX is believed to be involved in the detoxifying of photoproduced hydrogen peroxide. APX activity has been shown to increase in response to a number of stress conditions, such as drought, air pollution, high light intensity combined with chilling, UV light and deficiency in microelements.

[0007] So far, several different protein isoforms are known, those are two soluble cytosolic forms, and two chloroplast forms, one of which is stromal and the other thylakoid bound. Recently, several isoforms bound to membranes of glyoxysomes and peroxisomes were reported. Recently peroxisomal type ascorbate peroxidase was cloned from cotton (Bunkelmann & Trelease (1996) Plant Physiology 110, 589-598), *Arabidopsis* (Zhang *et al.* (1997) Plant Molecular Biology 34, 967-971) and spinach (Ishikawa *et al.* (1998) Plant and Cell Physiology 39, 23-34). Incidentally, novel gene according to this invention was obtained independent to known sequences described above.

[0008] Peroxisome, with glyoxysome, is an micro-organella exhibiting function, specialized to oxidative reaction utilizing molecular oxygen. These organellas described above are totally designated to microbody. The name of peroxisome is originated from production of hydrogen peroxide catalyzed by an enzyme localized at the organella. In the enzyme reaction, hydrogen atom is depleted from substrate, which is an organic compound, utilizing molecular oxygen. Moreover, catalase also exists in peroxisome. Hydrogen peroxide, generated by the reaction described above, is utilized for oxidation of compounds such as phenols, formaldehyde and alcohols by so-called "peroxidative reaction". Such oxidative reaction is involved in detoxification of

harmful molecules, accordingly exhibiting great importance especially in cells of liver or kidney.

[0009] Under various stress conditions, generation of hydrogen peroxide increases in microbody and the generated hydrogen peroxide diffuses into cytosol. The peroxisomal type ascorbate peroxidase of this invention is assumed to be bound outside of peroxisome membrane, operating to protect cells from injury by decomposition of hydrogen peroxide. Therefore, peroxisomal type ascorbate peroxidase of this invention would enable rapid deletion of active oxygen species generated in peroxisome before transition to cytosol, which could not be achieved by conventional ascorbate peroxidase of cytosolic form or chloroplast form.

[0010] These and other features and advantages of this invention will become apparent upon a reading of the detailed description and drawings.

BRIEF EXPLANATION OF THE DRAWINGS

[0011] Fig. 1 is a schematic view showing construction of pAPX over expression vector used to transform *Arabidopsis thaliana*.

Fig. 2 is a figure showing base sequence and deduced amino acid sequence of peroxisomal ascorbate peroxidase from barely.

Fig. 3 is a figure showing comparison of amino acid sequences of peroxisomal ascorbate peroxidase from barely, cotton and *Arabidopsis*.

Fig. 4 is a photograph showing Southern blot analysis for pAPX in barely using barely pAPX specific DNA sequence as a prove.

Fig. 5 is a photograph showing Northern blot analysis for pAPX expression in barely induced by heat and other treatments using barely pAPX specific DNA sequence as a prove.

Fig. 6 is a photograph showing PCR analysis of wild-type and

transgenic plants for the presence of pAPX transgene.

Fig. 7 is a photograph showing Northern blot analysis of wild-type plants and *pAPX*-overexpressing transgenic plants of T2 generation.

Fig. 8 is a graph showing comparison of wild-type and transgenic *pAPX* to heat stress, using fresh weight of green part in total leaf as the index.

Fig. 9 is a photograph showing comparison of growth between wild-type and transgenic *pAPX*, after exposure to heat stress.

DETAILED DESCRIPTION OF THE INVENTION

[0012] Therefore, the inventors have isolated peroxisomal ascorbate peroxidase gene from cDNA library prepared from barely leaf using the technique of differential display method. Moreover, the inventors have determined base sequence of the gene. As described above, all of peroxisomal ascorbate peroxidases cloned so far are those derived from dicotyledonous plants, such as cotton, *Arabidopsis* and spinach. The peroxisomal ascorbate peroxidase of this invention provides the first example of cloning peroxisomal ascorbate peroxidase derived from monocotyledonous plant. The differential display method utilized in this invention comprises the steps of RT-PCR using one short primer, separating by sequence gel and subsequently analyzing the obtained sequence. Here, said short primer consists of arbitrary sequence, exhibiting high frequency expression in the gene of eukaryotic cell. It should be noted that this method enables detection with high sensitivity, which is the advantage of this method. The base sequence of the gene of this invention was thus determined. Moreover, it was exhibited that expression of the gene was induced under high-temperature stress. Moreover, improved thermotolerance was also exhibited when the gene was over expressed in *Arabidopsis thaliana*.

[0013] This invention relates to peroxisomal ascorbate peroxidase gene originated from barley, consisting of a base sequence referred to as base

numbers from 1 to 1089 shown in SEQ ID NO: 2 in the sequence list. Said peroxisomal ascorbate peroxidase gene is involved in thermotolerance of a plant and its expression is induced by high-temperature stress.

[0014] According to technique of gene recombination, artificial modification can be achieved at a specific site of basic DNA, without alteration or with improvement of basic characteristic of said DNA. Concerning a gene having native sequence provided according to this invention or modified sequence different from said native sequence, it is also possible to perform artificial modification such as insertion, deletion or substitution to obtain gene of equivalent or improved characteristic compared with said native gene. Moreover, a gene with such mutation is also included in the range of this invention. That is, the gene, consisting of a base sequence hybridizes with said base sequence shown in SEQ ID NO: 2 in the sequence list under stringent condition, means a gene in which 20 or less, preferably ten or less, and more preferably five or less bases of the sequence is deleted, substituted or added to the base sequence shown in SEQ ID NO: 2 in the sequence list. Moreover, such gene exhibits homology 70% or more, preferably 80% or more and still preferably 90% or more with the base sequence shown in SEQ ID NO: 2 in the sequence list. In addition, such gene hybridizes with the base sequence shown in the SEQ ID NO: 2 in the sequence list under stringent condition. Such gene is also within the range of this invention so far as it exhibits the characteristic of peroxisomal ascorbate peroxidase gene, being induced by high-temperature stress.

[0015] Furthermore, this invention relates to peroxisomal ascorbate peroxidase polypeptide originated from barley, consisting of an amino acid sequence referred to as amino acid numbers from 1 to 291 shown in SEQ ID NO: 1 in the sequence list. The polypeptide is encoded by open reading frame portion of the base sequence indicated by SEQ ID NO: 2 in the

sequence list. The polypeptide consisting of an amino acid sequence in which a part of said polypeptide referred to as amino acid sequence shown in SEQ ID NO: 1 is deleted, substituted or added with another amino acid sequence means a polypeptide in which 20 or less, preferably ten or less, and more preferably five or less amino acids of the sequence is deleted, substituted or added to the amino acid sequence shown in SEQ ID NO: 1 in the sequence list. Moreover, such polypeptide exhibits homology 70% or more, preferably 80% or more and still preferably 90% or more with the amino acid sequence shown in SEQ ID NO: 1 in the sequence list. Such polypeptide is also within the range of this invention so far as it exhibits characteristic as peroxisomal ascorbate peroxidase polypeptide, being encoded by a gene induced by high-temperature stress.

[0016] A method to transform a plant by incorporating peroxisomal ascorbate peroxidase gene into a plant, and a transgenic plant produced by incorporation of said peroxisomal ascorbate peroxidase gene are also within the range of this invention. The peroxisomal ascorbate peroxidase gene of this invention is a gene induced by high-temperature stress and it is involved in self defense mechanism of a plant. Therefore, resistance to high-temperature stress can be rendered to a plant by incorporating said gene into a plant. The example of plants, preferred as a target plant, to which said gene induced by salt stress of this invention is incorporated, may include monocotyledonous plants, such as barely, wheat, lily, rice, maize and asparagus as well as dicotyledonous plants, such as *Arabidopsis*, tobacco, carrot, soybean, tomato and potato.

[0017] A conventional method known in this art, as a method to produce a transformant, can be utilized. A vector available in this invention may include a plasmid vector, such as pBI121 utilized in following embodiment and pBI221, but not limited to them. Such vector can be incorporated into an

Agrobacterium strain, then the target plant can be infected by said *Agrobacterium* to produce a transgenic plant. Moreover, transformed individuals can be selected using kanamycin resistance, incorporated into the plasmid, as a marker of selection. Furthermore, a seed from such transgenic plant can be obtained. The method to incorporate said plant gene of this invention is not limited to *Agrobacterium* method and other methods, such as particle gun method and electroporation method, can be also utilized for incorporation of the gene.

EMBODIMENT

(Plant material)

[0018] Barley (*Hordeum vulgare* L. Haruna-nijyo) plants were grown hydroponically with 1,000 times diluted Hyponex in tap water in a growth chamber under a light period of 13 h, 25°C, at 100 μmol $m^{-2}s^{-1}$, and a dark period of 11 h, at 23°C with the humidity at 70% for 2-3 weeks. For salt stress treatment, the NaCl concentration of the culture medium was raised in 100 mM steps every second day until 300 mM, and kept there for 2 d. Heat stress was conducted in the light by exposing the whole plants to 37°C with the humidity at 70% in a chamber for 24 h. Abscisic acid (ABA) was added directly into the culture solution at 50 μM and hydrogen peroxide at 1.5% (approximately 440 mM).

(Differential display)

[0019] Poly (A^+) RNA was prepared by using an oligo (dT)-cellulose column (Pharmacia). First-strand cDNA was synthesized by reverse transcriptase using random primers (6mer). PCR reactions were carried out as described previously by (Muramoto *et al.* Photosynthesis Mechanisms and Effects volume IV (eds Garab), pp.3043-3046. Academic Publishers). Salt-stress induced PCR fragments were

cloned into a pGEM-T vector (Promega). The DNA sequences were determined by the dye-primer sequencing method on a DNA sequencer (ABI: 373A). A partial fragment encoding part of APX was obtained, according to the BLAST homology searching.

(Cloning and sequencing of barley gene for pAPX)

[0020] A complementary DNA library of salt-stressed barley leaves was prepared as described previously (Ishitani *et al.* (1995) Plant Molecular Biology 37, 307-315). The library was screened by the PCR fragment labeled with [α-^{32}P] dCTP using a Megaprime DNA labeling system (Amersham). Positive clones were excised from the ZAPII vector into the pBluescript plasmid according to the manufacturer's protocol. The DNA sequences were determined as described above.

(Southern and Northern blot analysis)

[0021] Genomic DNA was isolated from barley leaves according to the conventional method. Genomic DNA was digested with restriction enzymes, BamHI, BglII, EcoRI, EcoRV, and HindIII, and separated on 0.8% agarose gel. After transferring to a nylon membrane (Hybond-N$^+$, Amersham), hybridization was carried out in a solution containing 6XSSC, 5X Denhardt's solution, 0.1% SDS and 0.1 mg/ml denatured herring sperm DNA. The membrane was washed in 2XSSC, 0.1% SDS, and then in 1XSSC, 0.1% SDS, and further in 0.1XSSC, 0.1% SDS. All hybridization and washing were performed at 65°C. Twenty-day-old plants were exposed to stress conditions as described above, and then harvested and stored at -80°C for RNA extraction. RNA was isolated by ATA (aurintricarboxylic acid) method. Twenty μg of total RNA were separated on 1.2% agarose gel containing 0.66 M formaldehyde and transferred to a

nylon membrane (Hybond-N, Amersham). All hybridization and washing were done at 65°C. The signals were analyzed with a BAS2000 Bioimage Analyzer (Fuji).

(Construct of expressing plasmid and transformation)

[0022] The *pAPX* sequence encoding the full-length pAPX protein was ligated into the binary vector of pBI121 with the replacement of the GUS fragment and under the control of the CaMV 35S promoter (Fig. 1). In Fig. 1, RB and LB indicates right border sequence and left border sequence, respectively. The plasmid was then introduced into *Agrobacterium tumefaciens* by electroporation. *pAPX* was introduced into *Arabidopsis thaliana* ecotype Columbia mediated via *Agrobacterium* by *in planta* transformation (Shimamoto K. and Okada K. ed. Experimental Protocol for Model Plants : Rice and Arabidopsis, SYUJUNSYA Co. Ltd, Tokyo, 1996). After harvesting, the seeds were plated on kanamycin-containing MS (Murashige & Skoog) medium and the kanamycin-resistant plants (T1) were collected. The transformed seedlings were transferred into vermiculite-containing pots watering with 1,000 times diluted Hyponex in tap water and grown under routine conditions for harvesting the T2 seeds.

(Thermotolerance test with transgenic plants)

[0023] The seeds of wild type and selected fully homozygous T3 transgenic lines were sown on vermiculite watering with 1,000 times diluted Hyponex in tap water and grown in a growth chamber under a light period of 16 h, 23°C, 100 μmol $m^{-2}s^{-1}$ and dark period of 8 h, 23°C with the humidity at 50% for 3 weeks. Plants were exposed to 35°C with the humidity 70% in the growth chamber for 5 d and then transferred to the routine conditions for recovery. After 5 d, the plants were harvested and the fresh weight of the green part and

yellow parts were weighed separately. Each experiment was done with five replicates.

(Cloning and Sequencing of *pAPX*)

[0024] By DDRT-PCR (differential display reverse transcription PCR) with a RAPD primer (5'-CTTGAGCGTATT-3'), the inventors detected a DNA fragment that was preferentially amplified from mRNAs prepared from the salt-stressed leaves. After sequencing, this fragment was found to have high homology to APX genes according to BLAST homology searching. Using this fragment as the probe, the inventors subsequently isolated the full-length cDNA clone from a cDNA library prepared from the leaves of salt-stressed barley. The cDNA was 1089 bp in length and contained an open reading frame encoding a predicted polypeptide of 291 amino acids (Fig. 2). In Fig. 2, the transmembrane region was indicated by underline and the characteristic long C-terminal was indicated as italic. Its deduced amino acid sequence had 75.3% homology to that from *Gossypium hirsutum* glyoxysomal APX (gAPX) and 72.1% homology to that from *Arabidopsis thaliana* APX3 (Fig. 3). Both the characteristic long C-terminal part and the transmembrane domain were also found and conserved. So, the isolated clone encodes the peroxisomal type ascorbate peroxidase and we designated it as pAPX.

(Southern Blot Analysis)

[0025] To estimate the copy number of the gene, we carried out Southern blot analysis using 3'-UTR plus 150 base pair (bp) of C-terminal encoding region as a probe. The pAPX cDNA has one internal HindIII restriction site but no site for BamHI, BglII, EcoRI, and EcoRV restriction enzymes. In Fig. 4, lanes 1, 2, 3, 4 and 5 indicate results of digestion by BamHI, BglII, EcoRI, EcoRV and

HindIII, respectively. As shown in Fig. 4, under high-stringency condition, only a single band in the BamHI, BglII, EcoRI and EcoRV digests and 2 bands in HindIII digest were detected. This indicates that pAPX was encoded by a single-copy gene in the genome of barley.

(Expression of *pAPX* genes under heat and other stresses)

[0026] Fig. 5 indicates result of Northern blot analysis of pAPX under various stress. In Fig. 5, samples were prepared from plants grown under normal (C), salinity (300 mM NaCl), ABA-treated (50 μM) conditions, and heat-stress (37°C, 24h). In upper photograph A, detection was performed using 3'-UTR of pAPX cDNA plus 150bps of c-terminal encoding region as a probe. In lower photograph B, 25S rRNA was visualized by EtBr, as a control. RNA blot analysis as a control (Fig. 5) indicated that pAPX transcript level was obviously increased under heat and salt stress. Addition of ABA also caused even higher induction of pAPX gene expression. However, adding hydrogen peroxide directly to the growth medium did not induce its expression.

(Thermotolerance of the Transgenic Arabidopsis overexpressing pAPX)

[0027] From the T0 seeds pool (harvested from *Agrobacterium tumefaciens* infected Arabidopsis plants), totally 8 independent transgenic lines (T1) were screened out from kanamycin-containing media. After self-pollinated, T2 progency were plated on kanamycin-containing media again for segregation analyses. According to the segregation analysis, six lines were found to have a single T-DNA insertion. The genomic DNAs were isolated from wild-type and six T2 transgenic lines, then analyzed by PCR using

two sets of primer of *pAPX* representing 131-511bp and 131-916bp. The result is shown in Fig. 6. In Fig. 6, PCR products representing pAPX sequence region of 131-916 bp is shown in the left and PCR products representing pAPX sequence region of 131-511 bp is shown in the right. In addition, pAPX1, pAPX2, pAPX3, pAPX5, pAPX7 and pAPX8 indicate each dependent Arabidopsis strains, respectively. No DNA fragment could be amplified from wild-type plants, whereas an expected 380 bp or 785 bp long fragment was amplified from DNAs of all transgenic lines tested (Fig. 6), indicating that these lines contain the *pAPX* transgene. T3 transgenic lines were produced by self-pollination of T2 again. From these, 50-60 seeds were plated on Kanamycin-containing MS media to select homozygous lines which showed all offspring resistant to kanamycin. Only those homozygous lines were used for further analysis and thermotolerance test.

[0028] The pAPX transcript level was checked by Northern blot analysis in both the wild type (WT) and transgenic *Arabidopsis thaliana* seedlings. Fig. 7 shows the result of Northern blot analysis of wild type plants and *pAPX*-overexpressing transgenic plants of T2 generation, using full-length pAPX as the probe. In upper photograph A, detection of transcripts hybridizing with the barely specific *pAPX* probe was performed on pAPX1, pAPX2, pAPX3, pAPX5, pAPX7 and pAPX8. In lower photograph B, 25S rRNA was visualized by EtBr, as a control. As shown in Fig. 7, the wild-type plants do not contain transcripts hybridizing with the *pAPX* probe, but all transgenic lines contain abundant pAPX transcript. Among them, lines of pAPX1, pAPX2 and pAPX3 had the highest level, while pAPX5 and pAPX7 were relatively lower. The pAPX expression was very weak in the line pAPX8.

[0029] When 3 weak-old plants were exposed to heat stress, all transgenic lines tend to be more tolerant to high temperature stress than wild type. The percentage of green part over the total in line pAPX5 and pAPX1 were significantly higher ($P<0.05$) than WT (Fig. 8). However, the thermotolerance is not positively correlated to the pAPX transcript level in the transgenic lines, since line pAPX5 which has lower transcript level was more tolerant to high temperature stress than pAPX1-3 with higher transcript levels. Moreover, Fig. 9 is the photograph exhibiting growth condition of plant bodies of wild type strain and pAPX transformants, after exposure to high temperature stress. Accordingly, it was revealed that transformants expressing suitable amount of pAPX mRNA exhibited significant thermotolerance. This may imply importance of the optimal balance among pAPX and other antioxidant enzymes. Fig. 9 shows that growth of transformed *Arabidopsis* was fairly good even after exposure to high temperature stress, indicating improved thermotolerance of the transformant. Compared with it, growth of wild type strain was damaged as shown in Fig. 9.

[0030] This method provides peroxisomal ascorbate peroxidase gene of barely, a novel gene induced by high-temperature stress. This invention also provides a transgenic plant exhibiting resistance to high temperature stress, produced by incorporating said gene. The method of this invention provides a novel technique to render thermotolerance to a plant.

Sequence listing

<110> President of Nagoya University

<120> Peroxisomal ascorbate peroxidase gene induced by high temperature stress and a transgeneic plant exhibiting thermotolerance

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<210> 1

<211> 291

<212> Amino acid

<213> *Hordeum vulgare* L. Haruna-nijyo

<400> 1

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SVCPREGRLP DAKKGAPHLR DIFYRMGLTD KDIVALSGGH SLGKAHPERS GFDGAWTRDP 180

LKFDNSYFLE LLKGESEGLL KLPTDKALLD DPEFRRYVEL YAKDEDVFFK DYAESHKKLS 240

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CTCCGCCTCG CATGGCATGA TGCTGGCACC TATGATGTGA ACACAAGAAC TGGTGGTGCA 180

AATGGTTCAA TTAGATACGA GGAAGAGTAC ACCCATGGTT CAAATGCTGG CTTAAAAATT 240

GCTATTGATC TCCTTGAGCC TATTAAAGCG AAGCATCCAA AGATTACATA TGCAGACCTT 300

CATCAGCTTG CCGGAGTAGT TGCAGTTGAA GTCACCGGGG GTCCAACCGT TGAGTTCATC 360

CCTGGAAGAC GTGATTCGTC AGTTTGTCCC CGTGAAGGAC GCCTTCCTGA TGCTAAGAAA 420

GGTGCACCAC ATCTAAGGGA CATCTTTTAT CGAATGGGGT TAACAGACAA AGATATTGTA 480

GCACTATCTG GGGGGCACAG CCTGGGAAAG GCGCATCCTG AAAGGTCTGG GTTTGACGGT 540

GCATGGACTC GTGACCCTCT GAAATTTGAC AACTCATACT TTCTTGAGCT ACTGAAGGGG 600

GAATCTGAGG GTCTTCTGAA GCTCCCTACT GATAAGGCAT TGTTGGATGA TCCTGAATTT 660

CGACGCTATG TGGAGCTTTA TGCAAAGGAT GAGGATGTTT TCTTCAAGGA CTACGCTGAA 720

TCACACAAAA AACTTTCTGA ACTTGGCTTC ACACCACGGA GCAGTGGCCC AGCATCTACA 780

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GCAGCTGTAG TTATCGCGGG CTACCTGTAC GAAGCTTCCA AGAGGAGCAA GTAAGGGGTT 900

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